HEMOPROTEIN DISCOVERY THROUGH PROTEOMIC TECHNIQUES

A Thesis
Presented to
The Academic Faculty

by

Cole Stapleton

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in the
School of Chemistry and Biochemistry

Georgia Institute of Technology
December 2018

COPYRIGHT © 2018 BY COLE STAPLETON

HEMOPROTEIN DISCOVERY THROUGH PROTEOMIC TECHNIQUES

Approved by:

Dr. Amit R. Reddi, Advisor
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Adegboyega Yomi Oyelere
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Neha Garg
School of Chemistry and Biochemistry
Georgia Institute of Technology

Date Approved: December 7, 2018

ACKNOWLEDGEMENTS

I would like to first thank Dr. Reddi. Getting to work with you has been a pleasure. The support and challenges you have provided throughout this project have helped me grow immensely as a scientist and thinker.

I also want to thank the entire Reddi lab. You all helped me in so many ways no matter what. The discussions we had, whether science related or not, always helped me get through a long day in the lab. One could only hope to work with people half as great as you all.

Finally, I would like to acknowledge my amazing family and friends. You all have been there through the good and bad times. I am really grateful for having such a supportive group of people on my side who have helped keep me sane and motivated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

BLH	Bioorthogonally labeled heme
Yeast	*Saccharomyces cerevisiae*
ALA	δ-aminolevulinic acid
ALAS	δ-aminolevulinic acid synthase
PBG	Porphobilinogen
HMB	Hydroxymethyl bilane
UROgenIII	Uroporphyrinogen III
CPgenIII	Coproporphyrinogen III
ALAD	δ-aminolevulinic acid dehydratase
PBGD	Porphobilinogen deaminase
UROS	Uroporphyrinogen III synthase
UROD	Uroporphyrinogen III decarboxylase
PPgenIX	Protophoryrinogen IX
PPIX	Protophyrin IX
CPOX	Coproporphyrinogen III oxidase
PPOX	Protophyrin IX oxidase
FECH	Ferrochelatase
Mfrn	Mitoferrin
OGC	2-oxoglutarate carrier
ABCB6	ATP-binding cassette B6
HRG	Heme responsive gene
FLVCR	Feline leukemia virus subgroup c receptor

DCC	Dicyclohexylcarbodiimide
PEG	Polyethylene glycol
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
UV/vis	Ultra violet/visible light spectroscopy
SA	Succinylacetone
EV	Empty vector
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

SUMMARY

Heme is an essential cofactor and signaling molecule implicated in a variety of diseases. For this reason, understanding how heme is transported and trafficked within many contexts would greatly help us in these health-related aspects. In order to better understand heme homeostasis, it is important to build a complete description of heme binding proteins. Proteomic techniques have been used to determine the heme binding proteome using heme affinity chromatography. One downside to this is that enrichment methods used often contain many non-specifically binding proteins, due to the hydrophobic properties of heme. To improve upon these enrichment methods, use of bioorthogonally labeled heme (BLH) can allow for hemoproteins bound to this BLH to be enriched from cells. This can enable the cell to incorporate the labeled heme in a physiologically relevant way. In this work, a variety of BLH were characterized and tested in *Saccharomyces cerevisiae* to prove the efficacy of this novel enrichment method. Studying these BLH both *in vivo* and *in vitro* has given us a better idea of how to implement them to discover hemoproteins in a wide range of contexts, which were previously unknown. This approach could greatly expand our ideas of the hemoproteome in organisms already being studied as well as more difficult organisms which are not genetically tractable.

CHAPTER 1. INTRODUCTION

1.1 Heme Functions and Biology

Heme b (iron protoporphyrin IX) is an essential cofactor and signaling molecule that helps facilitate a wide range of biological processes. These functions include gas binding, electron transport and catalysis.[1–5] Due to its utilization in such a diverse manner, heme can be found in virtually every compartment of the cell. Despite being essential for so many roles within the cell, heme is also cytotoxic due to its hydrophobicity and ability to produce reactive oxygen species.[6,7] These facts necessitate that heme concentrations and bioavailability are tightly regulated.[1–3,5] Misregulation of heme can be linked to diseases such as cardiovascular disease, neurodegenerative disease, and certain cancers.[5,8–11] The extent of our knowledge around heme homeostasis is mainly pertaining to heme synthesis and the physiology around tight binding hemoproteins. Heme trafficking on the other hand is not well understood.

Heme synthesis begins and ends in the mitochondria, from which it can be trafficked to populate a wide range of hemoproteins. This is done through a highly conserved eight-step pathway known as the Shemin pathway. Succinyl-CoA and Glycine are first converted to δ-aminolevulinic acid (ALA) in the mitochondria. ALA is then trafficked to the cytosol, where the next four steps convert 8 equivalents of ALA to coproporphyrinogen III. Coproporphyrinogen III is then transported back to the mitochondria for the final three steps, the final step being the insertion of iron into protoporphyrin IX to form heme.[12–14] From the matrix of the mitochondria heme is then transported to the mitochondria

intermembrane space, cytosol, endoplasmic reticulum, and nucleus to populate the cells hemoproteins.

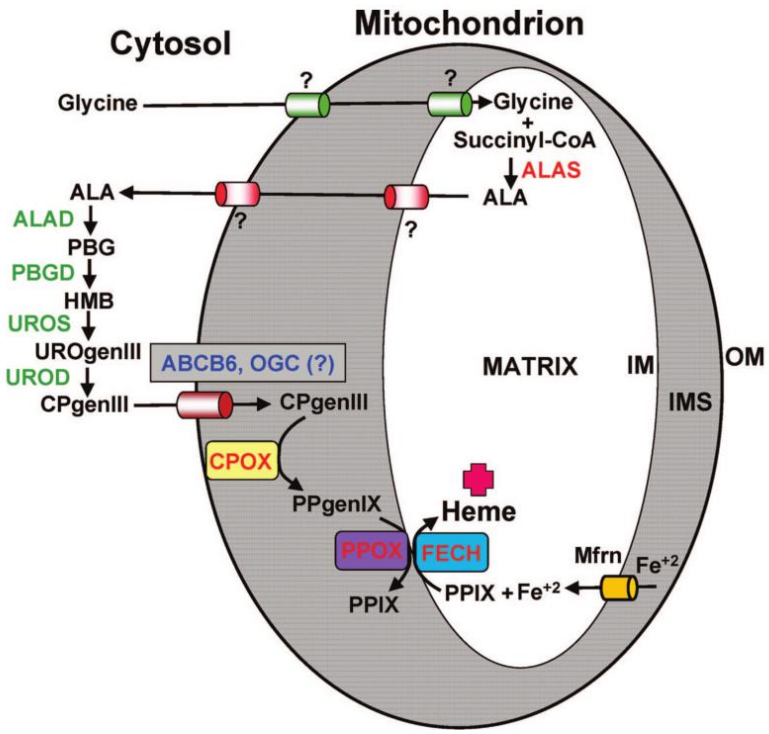

Figure 1: Heme biosynthetic pathway. The biosynthesis of heme is shown to occur in both the cytosol and mitochondria through an eight-step pathway. Glycine and succinyl-CoA are converted to ALA through ALA synthase (ALAS). ALA is then trafficked out of the cell and converted to porphobilinogen (PBG), hydroxymethyl bilane (HMB), uroporphyrinogen III (UROgenIII), and finally coproporphyrinogen III (CPgenIII) through 4 enzymatic steps involving ALA dehydratase (ALAD), PBG deaminase (PBGD), uroporphyrinogen III synthase (UROS), and uroporphyrinogen III decarboxylase (UROD). CPgenIII is then imported into the mitochondria where it is converted to protophoryrinogen IX (PPgen IX), protophyrin IX (PPIX) and finally heme through coproporphyrinogen III oxidase (CPOX), protophyrin IX oxidase (PPOX), and ferrochelatase (FECH). Mitoferrin (Mfrn) is involved in trafficking ferrous iron to the matrix of the mitochondria, while 2-oxoglutarate carrier (OGC) and ATP-binding cassette B6 (ABCB6) may be involved in the transport of CPgenIII into the inner membrane space. Reprinted (adapted) with permission from Severance, S.; Hamza, I. *Chem. Rev.* **2009,** 109(10), 4596-4616. Copyright 2009 American Chemical Society.[1]

While heme biosynthesis is well understood, there is very little known about the transport and trafficking of heme. As shown in Figure 2, heme is trafficked to each

compartment through pathways that are yet to be determined. The information we do know has been pieced together from various eukaryotic models. To name a few, two putative plasma membrane localized heme importers, heme responsive gene 1 and 4 (HRG-1 and HRG-4), two putative heme exporters, feline leukemia virus subgroup c receptor 1a and 1b (FLVCR1a and FLVCR1b), which are plasma membrane and mitochondrial localized, have been identified.[1,15] HRG-1 and its paralogue HRG-4 were first discovered in *C. elegans* through genetic screens, while only HRG-1 is found in the human genome. FLVCR 1 and 1b were shown to export heme in various mammalian models. As for chaperones and heme binding proteins, only a handful have been determined, HRG-2, HRG-3, glutathione s-transferase, fatty acid binding protein, and glyceraldehyde phosphate dehydrogenase (GAPDH) to list a few which are localized to the cytosol. HRG-2 is a membrane bound protein which facilitates in heme utilization by extraintestinal tissue while HRG-3 has been studied extensively in *C. elegans* to show that it is excreted in order to facilitate heme delivery within the intestines.[1,2] GAPDH has also been shown to buffer heme under various conditions in yeast models. [3] Many of the other putative heme chaperones have been determined based on their heme binding abilities, though their roles *in vivo* are not fully determined.[1,2] Thus, developing tools to better understand heme trafficking is essential to understanding diseases in which heme homeostasis is misregulated.

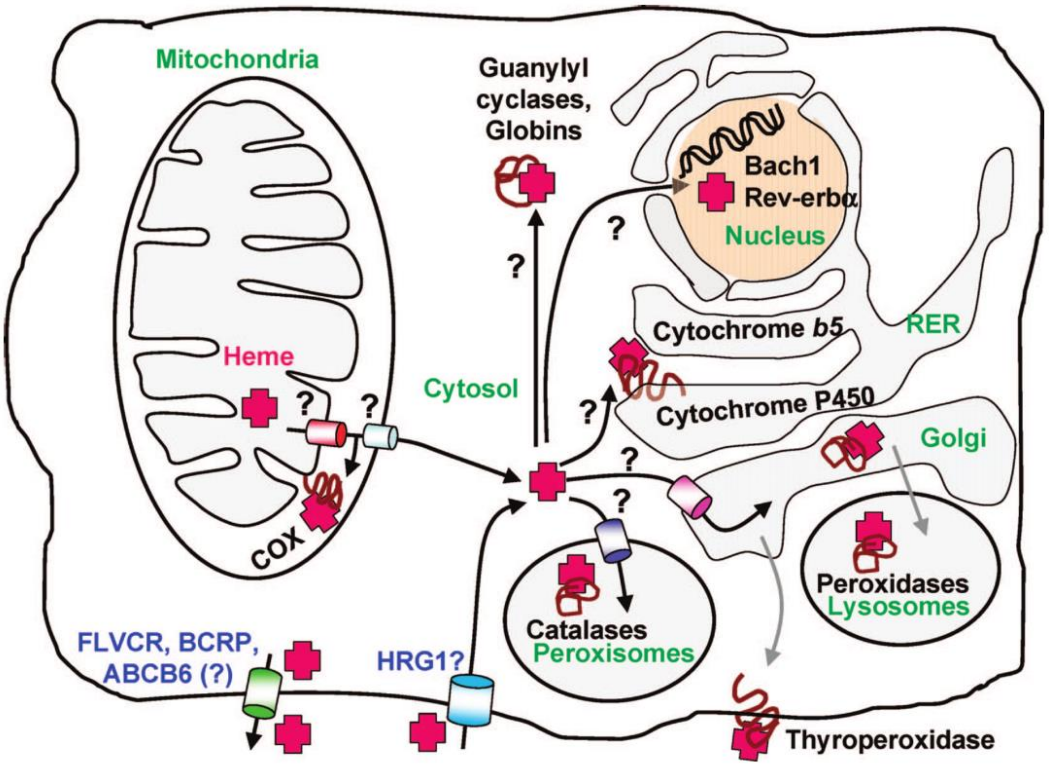

Figure 2: Schematic of heme trafficking pathways. Arrows with question marks (?) indicate unknown trafficking factors and potential hemoproteins of interest, while heme is represented by the red crosses. Some of the known factors involved in heme trafficking are listed in blue: feline leukemia virus subgroup C receptor (FLVCR), breast cancer resistance protein (BCRP), ABCB6, and heme responsive gene 1 (HRG1). Reprinted (adapted) with permission from Severance, S.; Hamza, I. *Chem. Rev.* **2009,** 109(10), 4596-4616. Copyright 2009 American Chemical Society.[1]

1.2 Current Hemoprotein Discovery Methods

Many genetic and biochemical approaches have been developed to identify protein and pathways involved in regulating heme homeostasis. Genetic approaches typically involve creating mutant cell lines by chemical mutagenesis, targeted gene deletion, or gene over-expression and screening mutants for heme-dependent phenotypes, including growth under heme deficiency or surplus, or screening the activity of heme enzymes. For example, observing the activity of catalase, cytochrome P450 enzymes, or heme-dependent

transcription factors can determine how specific mutations impact heme dependent processes and have the potential to reveal new heme transport and trafficking factors that dictate heme availability.[4,16,17] However, given that heme synthesis is impacted by many factors, including iron, zinc, and copper homeostasis, as well as oxygen availability and sensing, it is often difficult to parse heme synthesis from heme trafficking using genetic screens.

Biochemical approaches towards identifying heme trafficking factors complement genetic approaches quite well. Most commonly, hemin-agarose affinity chromatography is employed to enrich hemoproteins from complex cell and tissue extracts. The enriched hemoproteins are then chromatographed by SDS PAGE, isoelectric focusing, and/or ion exchange chromatography, followed by mass spectrometry to identify novel heme binding proteins.[18–20] Two major downsides that come with this technique are that 1) many non-specific binders are typically pulled out of solution and 2) candidate proteins may not bind heme *in vivo* in a physiologically meaningful way. Reducing this background of non-physiological heme proteins could help give a more meaningful list of putative hemoproteins that could then be characterized through more direct methods.

The Reddi lab has pioneered new integrated genetic and biophysical approaches towards characterizing heme homeostasis. The lab has developed genetically encoded ratiometric fluorescent sensors for heme that can image and quantify bioavailable heme. Using these probes in combination with genetic screens, the Reddi lab is systematically identifying new heme homeostatic factors and physiological conditions that regulate heme bioavailabilty.[4,5] Despite the utility of traditional genetic and biochemical approaches, and the development of new fluorescent probes for heme, more tools are needed to dissect heme

homeostasis. This work is focused on the development of new azido-heme analogs that can be utilized to enrich and identify novel hemoproteins in a variety of cell types.

1.3 Background and Chemistry

The development of bioorthogonal labels has been instrumental in many different contexts. Often times used to label substrates or metabolites, bioorthogonal labels are frequently used to enrich the modified proteins which have allowed the labeled substrate or ligand to be incorporated into the protein.[21–24] In this work, a bioorthogonal label, azide, was attached to heme and used to enrich proteins that interact with the bioorthogonally labeled heme (BLH). Feeding cells BLH can allow the incorporation into proteins through the use of the cellular machinery. Subsequent enrichment of proteins bound to the labeled heme can then be done through the method described.

The synthesis of BLH follows a well-developed, one pot amide bond formation reactions. Carbodiimide reagents have been used to form an amide bond between carboxylates and amines.[25] These reagents allow for the carboxylate to be activated, such that it can be attacked by the nucleophilic amine and result in the formation of an amide bond. The first step in Figure 3 shows how dicyclohexylcarbodiimide (DCC) can be used to append 3-azido-1-propanamine to one of the carboxylates of heme, catalyzed by hydroxybenzotriazole (HOBt). This was applied with other reagents containing differing length polyethylene glycol (PEG) linkers as well shown in the bottom pathway of Figure 3.

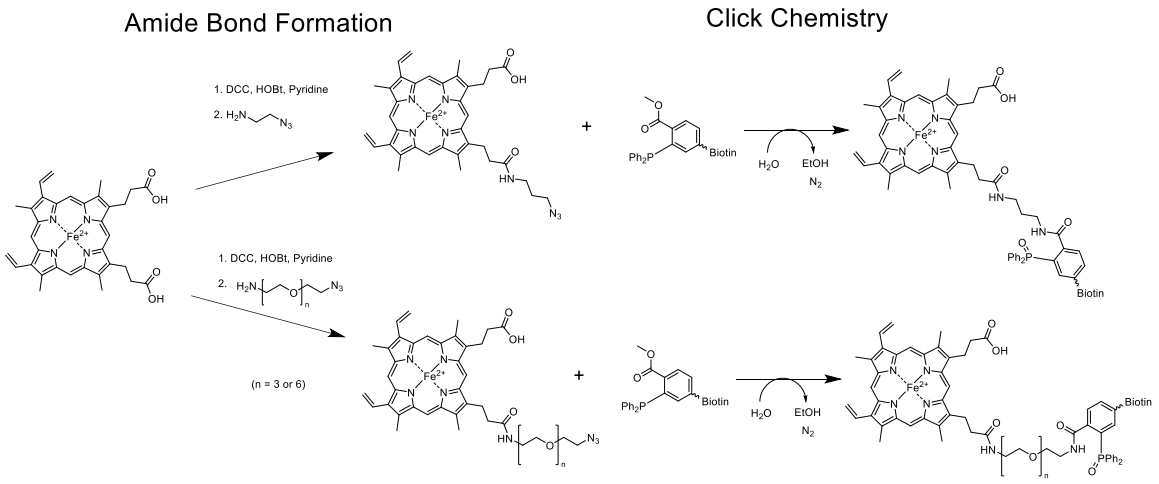

Figure 3: The two chemical modifications made to heme throughout the studied method of hemoprotein enrichment. The first step, Amide Bond Formation, is done in a one pot reaction and then purified for subsequent use. The addition of dicyclohexylcarbodiimide under basic conditions allows for the activation of the carboxylates of heme. The addition of amine linked to azide then allows the amine group to form a peptide bond with the activated carboxylate, while catalyzed by HOBt. In the second step, the azide group is then able to react with phosphine linked to biotin through Staudinger ligation, a type of Click Chemistry.

Once appended, the bioorthogonal azide can be reacted with a phosphine reagent through Staudinger ligation to covalently link a functional group to it. The Staudinger ligation can allow for an affinity label to be added to the bioorthogonal handle and then be enriched.[26–29] In the second step of Figure 3, the azide of the BLH is reacted with phosphine PEG-3 biotin covalently attaching the biotin group to heme. The biotin labeled heme can be pulled on with solid phase bound streptavidin. This type of affinity label is commonly used due to the affinity of biotin and streptavidin being the highest known noncovalent interaction.[30] This technique results in the enrichment of any proteins associated to the labeled heme.

1.4 Overview of Enrichment Method

To improve upon currently inefficient affinity purification methods, we use bioorthogonal azide labels to enrich hemoproteins from cells. The method utilized allows the cells to incorporate BLH through use of the cellular machinery, the steps of which are shown in the schematic of Figure 4. By feeding BLH, cells can uptake and incorporate BLH into its hemoproteins. Once lysed, Staudinger ligation appends biotin, which can then be enriched with solid-phase bound streptavidin. Proteins can then be eluted and analyzed by SDS-PAGE gel imaging and mass spectrometric approaches. The subsequent chapters will show how this method can be effectively implemented.

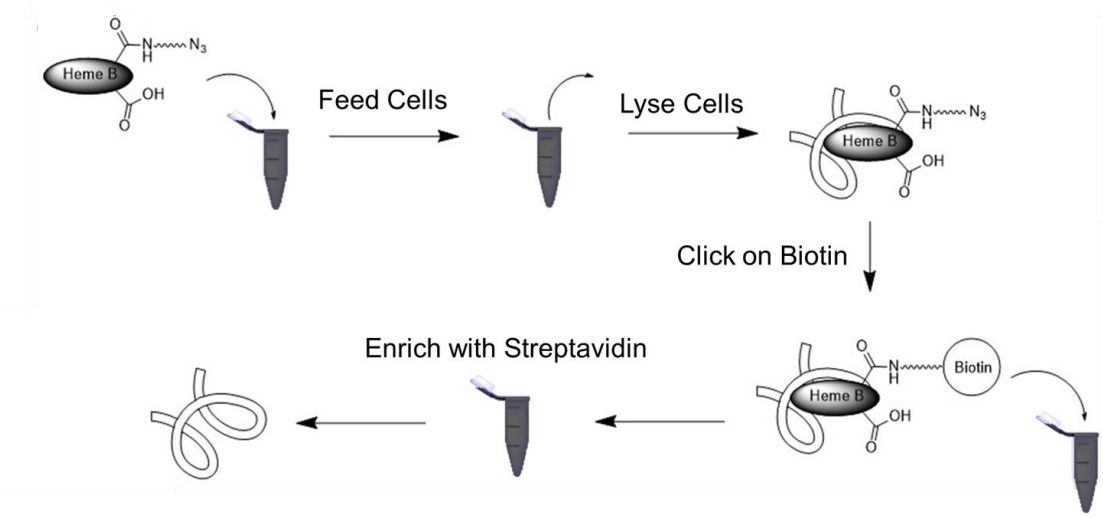

Figure 4: Schematic of hemoprotein enrichment from cells using BLH. In this work flow, BLH are fed to cells. The cells can then be lysed and biotin appended through click chemistry described above. Proteins bound to the BLH can then be enriched with solid phase bound streptavidin. These proteins can then be eluted and further studied.

CHAPTER 2. SYNTHESIS AND CHARACTERIZATION

2.1 Introduction

In our approach of hemoprotein discovery, we used a bioorthogonal label, azide, to enrich heme binding proteins. Bioorthogonal labeling with azides have been effectively used in many different proteomic approaches, often to look at labeled substrates or metabolites which get incorporated into proteins.[21–24] A major difference in our approach is that our label is not covalently incorporated into the protein. For this reason, testing how the modification impacts many properties in comparison to heme is essential to determine if bioorthogonally labeled heme (BLH) will be effective in hemoprotein enrichment. We also needed to try a variety of different BLH to see if differences in linker length could improve the properties of the BLH. With these various parameters, we were able to characterize each and determine which analog to use in further studies in cells. To start we synthesized and purified each compound and then were able to test a variety of properties *in vitro* that each BLH had.

2.2 Synthesis and Purification of Heme Analogs

First, we needed to append the bioorthogonal label to heme and purify it before we could determine the efficacy of this method. As shown in Figure 3, heme was converted to BLH with various linker lengths by amide bond formation at one of the carboxylates of heme. In designing this synthesis of the BLH, the two major considerations which led to the addition to the carboxylate were the fact that the carboxylates are not conjugated to the aromatic porphyrin of heme and the chemistry of the step is well understood.

In order to create these BLH, heme was reacted with azido amines with various linker lengths. This was done by appending the amine to the heme carboxylate as shown in the first step of Figure 3. In each case, DCC and HOBt were used to facilitate this reaction under basic conditions. DCC is known to activate the carboxylate, allowing for the amine to attack, while HOBt catalyzes the reaction. This was done with 3-azido-1-propanamine, azido-PEG3-amine, and azido-PEG6-amine to form azido-propyl-heme, azido-PEG3-heme, and azido-PEG6-heme, our three BLH.

To both monitor the reactions and do subsequent purification, reverse phase high performance liquid chromatography (HPLC) was used. Heme has a strong absorbance at 400nm, known as the Soret band, making it easy to be observed by HPLC coupled to UV/vis detection. This was also true with the analogs generated through this synthesis, as will be shown in Chapter 2.3 Characterization of Heme Analogs. The addition of the azido amines was observed to change the retention time due to the change in hydrophobicity. In Figure 5, three major peaks formed from this reaction, though the heme peak observed at 6.9 minutes was small due to depletion. The peak at 9.1 minutes was attributed to addition of a single azido amine per heme molecule, while the peak at 11.2 minutes was attributed to the addition of two azido amines per heme molecule. These analytes were collected during preparatory scale HPLC and confirmed by mass spectrometric analysis. In Figure 6, the major peak at 816.3 Da corresponds with azido-PEG3-heme shown in Figure 3. The purified product shown in Figure 5 was found to be around 95% purity. Other samples ranged from 80-95% pure after purification, with mainly heme impurity. Further characterization was done using these purified products containing a single azido amine per heme shown in Figure 3.

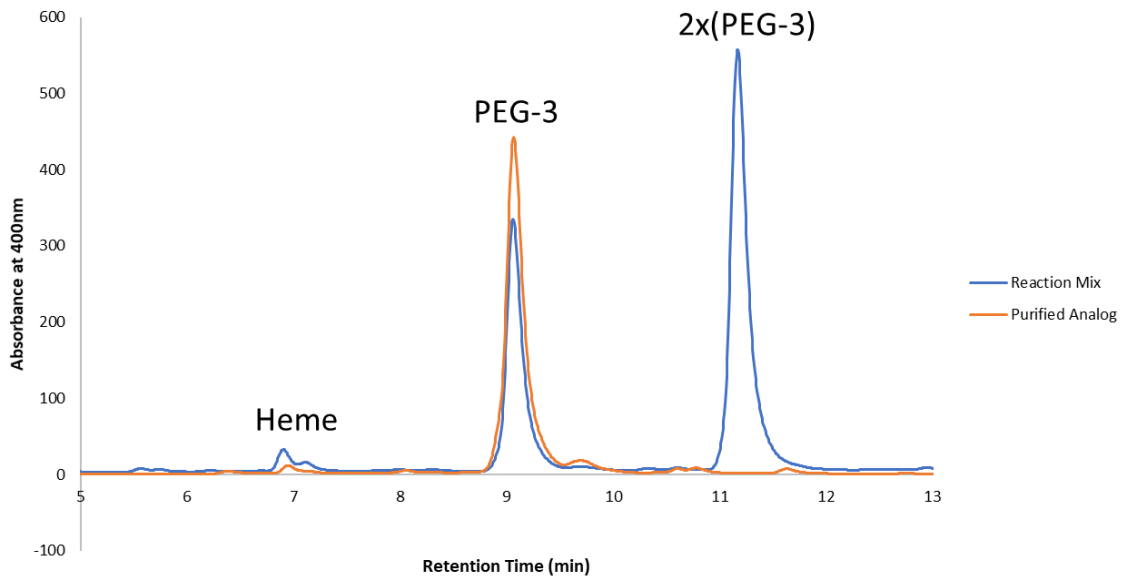

Figure 5: The synthesis and purification of the BLH shown by HPLC. The formation of BLH was observed by a shift in retention time under reverse phase HPLC. Equal moles of heme, DCC, HOBt, and azido-PEG3-amine were reacted together. The reaction mix was then purified by preparatory scale HPLC. The reaction mix is shown in blue, while the purified product is shown in red. This shows the resulting purification of the PEG3 linked heme. The product caused by two azide PEG-3 amine molecules reacting with heme ('2x(PEG-3)') can be separated through this process.

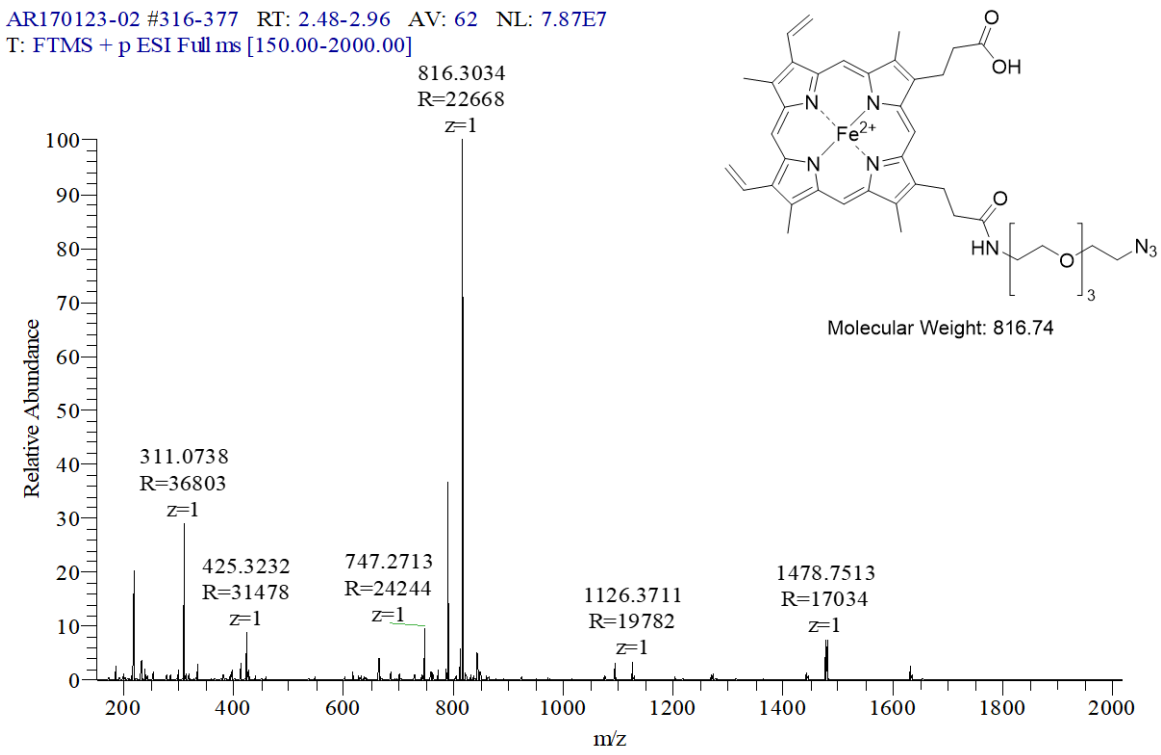

Figure 6: Electrospray mass spectrometric data of purified BLH. PEG3 linked heme was expected to have a mass of 816.7 Da. The peak corresponding to this mass is observed, showing that PEG3 linked heme was formed.

2.3 Characterization of Heme Analogs

When covalently attaching a bioorthogonal label, a large consideration is how this will impact the properties of the molecule. In our case, some major features of heme which could be impacted are the electronics of the porphyrin, the change of a negatively charged carboxylate to an amide, and the addition of a large group which could prevent protein binding. From the aspect of the bioorthogonal label, it is also important to think about the accessibility of the label as this can impact the ability of the analog to be enriched from solution. The characterization done addressed these questions and further allowed us to determine which to use in cell studies.

2.3.1 Spectral Characterization

Does the appended azide significantly change the electronics of our proposed molecules? This was important to determining whether hemoproteins would still bind to BLH the same way as heme. Due to the fact that the carboxylate being altered is separated by three unconjugated carbons, we did not expect the electronic properties to change drastically from this modification. Despite this, there still existed other possible contributors that could have impacted the electronics of the BLH. From the result shown in Figure 7, it does not seem to change very much spectroscopically, as seen through UV/vis spectroscopy. The heme-like characteristics were determined for each of the azido-heme analogs and seem to be relatively similar to heme independent of linker length. Though this does not mean binding may be impacted, further characterization was done to determine this effect.

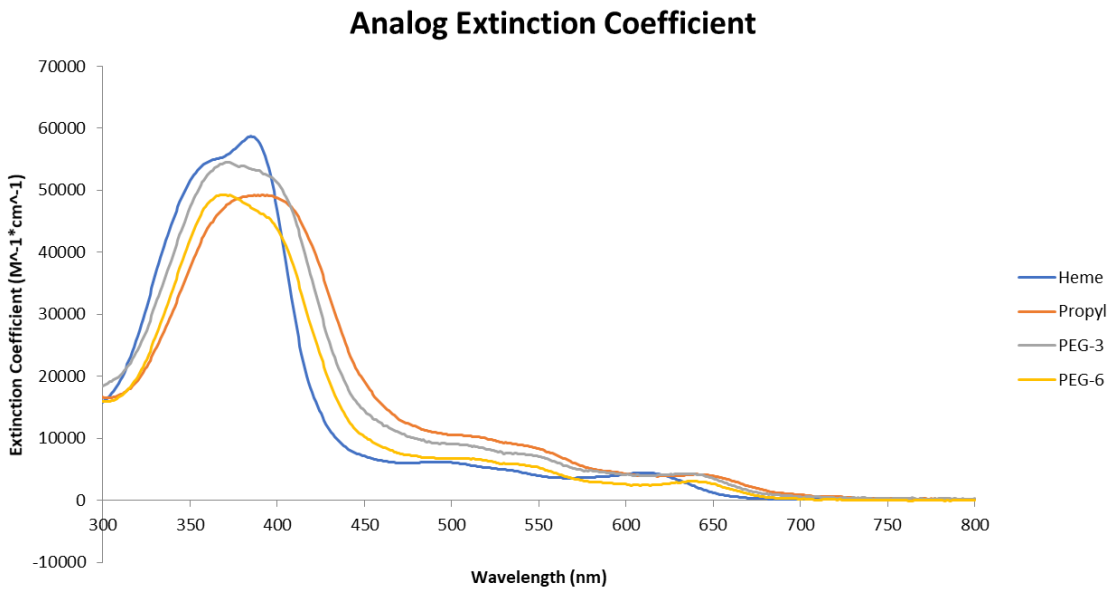

Figure 7: UV-visible absorbance spectra of each BLH. Similarities between each spectrum indicated that the addition of azide to the carboxylate of heme did not greatly impact the electronic of the molecules. Extinction coefficients were determined by serial dilution from 30 uM to 5 uM in deionized water at pH 7. BLH spectra are named 'propyl', 'PEG-3', and 'PEG-6' based on the linker of each molecule.

2.3.2 Monitoring Click Reaction

Once the azide was appended to heme to form BLH, it was important to test that the Staudinger ligation could occur under conditions compatible with cell lysates. In this reaction, each of the BLH were incubated with phosphine-PEG3-biotin, which is shown in the second step of Figure 3. Data from this reaction can be seen by HPLC chromatograms in Figure 8. In each chromatogram impurity heme could be seen at 7.3 minutes. Peaks corresponding to the BLH could be seen at 9.7, 9.5, and 9.4 minutes for each of the propyl (Figure 8a), PEG3 (Figure 8b), and PEG6 (Figure 8c) linked heme respectively. In Figure 8a, the propyl linked heme was consumed as the reaction proceeds, while two peaks at 8.8 and 9.0 minutes appear. These resulted from product of the Staudinger ligation, in which

biotin is added to the azide handle. In the case of PEG3 linked heme, the starting material was nearly completely consumed as shown in Figure 8b, while a new peak formed at 12.2 minutes, corresponding to the Staudinger ligation product. Finally, the PEG6 linked heme appeared to only react under these conditions slightly, forming a small peak for the Staudinger product at 11.4 minutes. From these results, it appeared that the reaction occurred rapidly for the propyl and PEG3 linked heme. The reaction did not seem very efficient for the PEG6 linked heme. This could be due to a number of factors such as aggregation and accessibility of the azide. But this does not discount any of the analogs because they may interact very differently with the cell's uptake machinery and proteins.

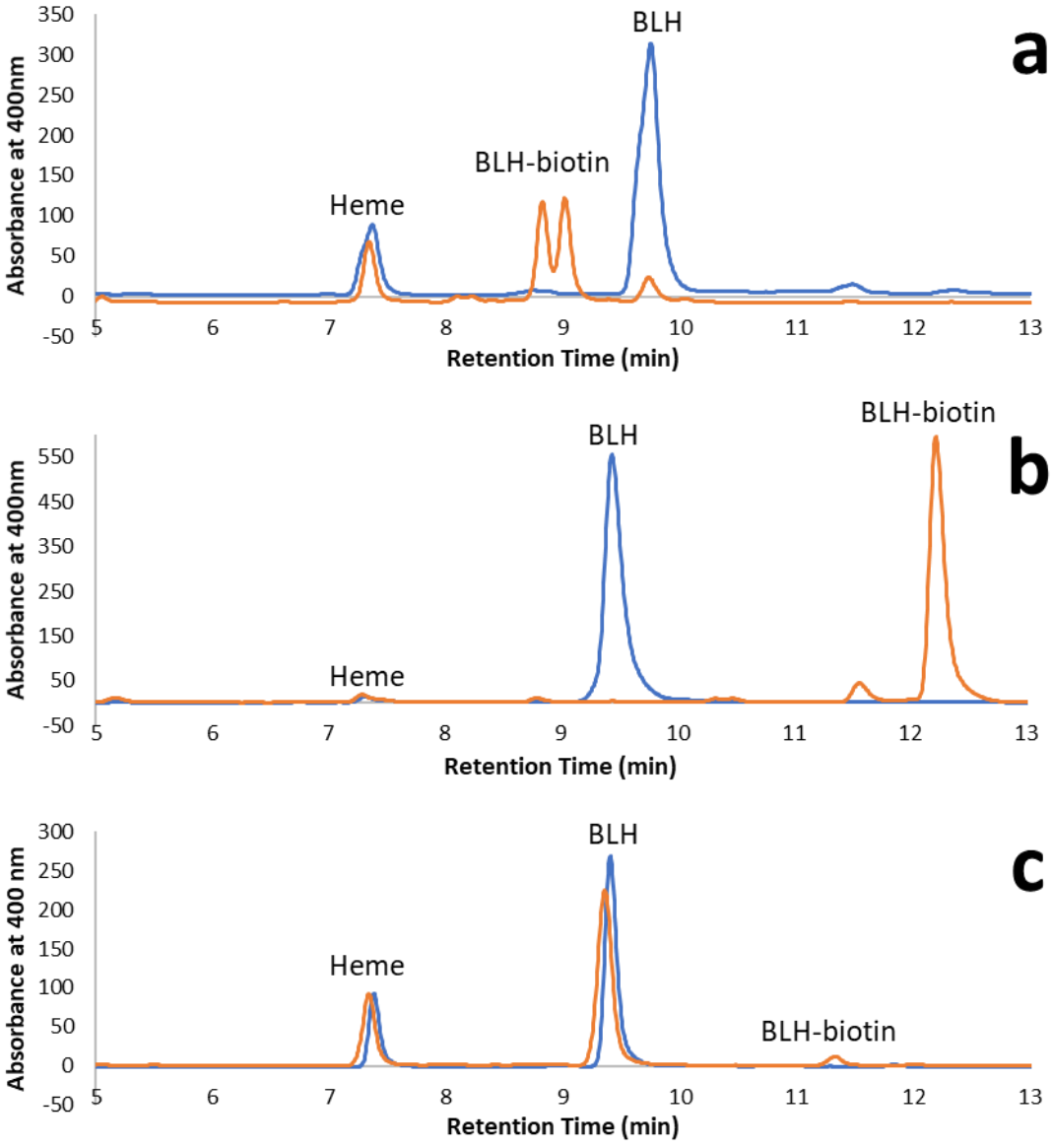

Figure 8: Monitoring Staudinger ligation reaction by HPLC. 10 uM of each BLH with propyl (a), PEG3 (b) or PEG6 (c) linker were incubated with 100 uM phosphine-PEG3-biotin in PBS for 3 hr at 37ºC. Chromatograms in red were the BLH incubated with a 10-fold excess of phosphine-PEG3-biotin, while chromatograms in blue are unreacted BLH. The change in peaks labelled BLH shows the extent of reaction over this time.

2.3.3 Model Hemoprotein Enrichment

Though the modifications to heme did not seem to impact the electronics of the conjugated porphyrin significantly, it could still impact how hemoproteins bind and interact. Seeing how a model hemoprotein interacts with the BLH can help us understand how this modification impacts binding. Thus, we tested the ability of each BLH to enrich the model hemoprotein myoglobin. To do this, apo myoglobin was incubated with each of the BLH, propyl, PEG3, and PEG6 linked heme, along with a heme control. Each were incubated either with or without phosphine-PEG3-biotin. Then the samples were incubated with streptavidin bound to magnetic beads. These were then be washed and bound proteins eluted. The eluted myoglobin was then run on SDS-PAGE gel. As seen in Figure 9, the heme control samples were unable to enrich myoglobin because they lacked the azide, and therefore lacked biotin. In each of the cases of BLH without phosphine-PEG3-biotin, the samples were also unable to enrich myoglobin. However, in the presence of phosphine-PEG3-biotin, each BLH was able to enrich myoglobin from solution, meaning they were able to bind to myoglobin. The most enrichment occurred from the PEG3 linked heme, while very little was seen from the propyl and PEG6 linked heme. It is difficult to discern whether the difference is due to effectiveness of binding or efficiency of the Staudinger ligation. In the case of PEG6 linked heme, very little was enriched most likely because the Staudinger ligation was not very efficient under these conditions. Overall each BLH was able to enrich myoglobin to some extent but it was still difficult to tell which would function best in cells. This is once again due to the fact that the uptake machinery and hemoproteins may interact with each BLH very differently *in vivo*. Therefore, testing each

BLH within cells would be beneficial further understanding the efficacy of these BLH analogs.

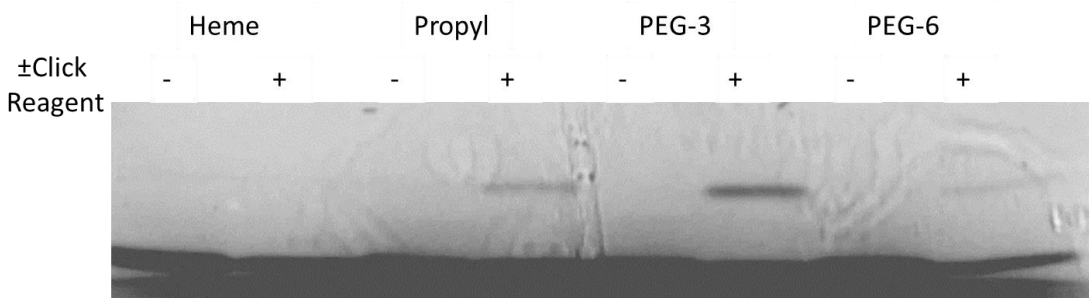

Figure 9: Myoglobin enrichment from PBS solution using BLH. Relative quantities of myoglobin enriched from each BLH based on band intensity shown by SDS PAGE gel stained with Coomassie. 10 uM of each BLH was incubated with 0.2 mg/ml apo-myoglobin and 100 uM phosphine-PEG3-biotin for 3 hr at 37ºC in 100 uL PBS. Samples were then incubated with 50 uL streptavidin mag sepharose bead slurry before washing and eluting bound myoglobin with SDS at 100ºC. BLH samples used are labelled 'propyl', 'PEG-3', or 'PEG-6' based on the linker of each.

CHAPTER 3. IN CELL STUDIES OF HEME ANALOGS

3.1 Introduction

The ability of cells to take up bioorthogonally labelled heme (BLH) was a major aspect of the method we developed. For this reason, it was important to test ways to improve uptake and potentially improve our ability to enrich hemoproteins from cells. To test the feasibility of the BLH *in vivo*, a variety of experiments were performed to test the ability of cells to uptake the BLH and incorporate them into cells. *Saccharomyces cerevisiae*, baker's yeast, was chosen due to its ease of use and easily accessible libraries of yeast knock out strains. One issue with using wild type yeast cells is that their ability to take up exogenous heme is poor.[31,32] For this reason, we attempted to improve the uptake of our BLH in yeast through a few different methods. In each case we were able to improve BLH uptake. Once it was determined that the BLH could get into cells and incorporate into proteins, enrichment from cells fed BLH was done.

3.2 Cellular Uptake of Heme Analogs

Despite poor heme uptake in wild type yeast cells, there are a number of ways to improve heme uptake in yeast cells. The hope in this work was to both determine uptake of the BLH and optimize it, such that the enrichment from cells was most effective. In these experiments we fed various amounts of either heme or BLH and determined quantities of uptake by HPLC. Much of the heme or BLH found in cells was localized to the cell wall. In application, this heme would not be very significant to our enrichment of soluble proteins from cells. Thus, we looked at heme extracted from cell lysates, rather than total

cellular heme. To do this, we lysed cells using an aqueous solution with detergent. The soluble proteins were separated from the insoluble cell debris, which contained much of the heme and BLH. Then acid acetone was used to extract heme and BLH from the cell lysates. These extracts could then be run on HPLC to quantify intracellular heme and BLH. Under various conditions we could then determine the optimal way to feed these BLH to cells.

It has been shown that heme depleted cells uptake heme better than heme replete cells.[32] Thus, we began by testing if these conditions could also improve uptake of our BLH. To deplete our cells of heme, we fed wild type cells succinylacetone (SA). SA is a known inhibitor of ALA dehydrogenase which is the second step in heme biosynthesis, as seen in Figure 1. In Figure 10a, it is seen in the blue trace that no detectable heme is produced by yeast under SA treatment, while those grown normally produce a small amount of heme, as seen by the peak at 7.8 minutes. From Figure 10b, it shows that more heme is found under SA treatment than without SA treatment. This corroborates the point that heme depleted cells are more capable of taking up heme.

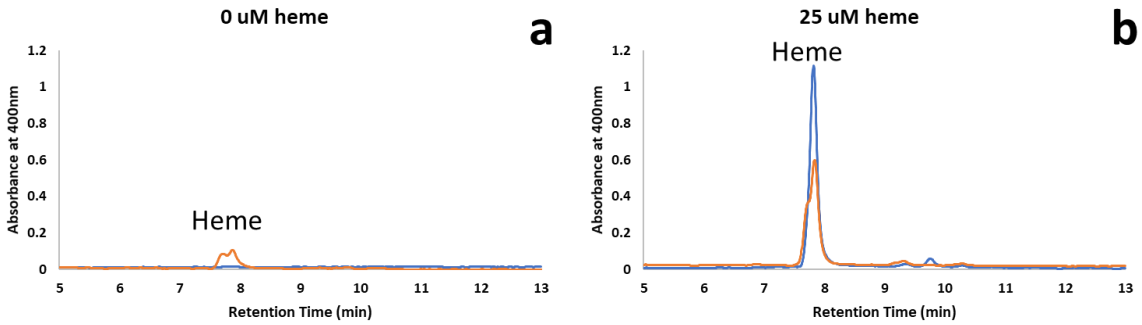

Figure 10: Heme uptake under heme depleted and heme replete conditions shown by HPLC. Heme fed to yeast cells was extracted from soluble lysates with acid acetone (5 mM HCl). Yeast cells were grown up in the heme biosynthetic inhibitor succinyl acetone (SA) overnight prior to feeding. Yeast cells were fed 0 uM heme (a) and 25 uM (b), either under heme deplete conditions fed 500 uM SA (blue) or under heme replete conditions without SA (red).

While testing BLH uptake with yeast cells under heme deplete conditions, we noticed slightly increased uptake in BLH, for the PEG3 and PEG6 linked heme. However, these increases were not very significant. This was seen from the chromatograms in Figure 11b and 11c by the slight increase from red to blue, representing the change in BLH from heme replete to heme deplete cells respectively. For the PEG3 and PEG6 linked heme this was seen by the peaks both around 9.8 minutes. As for the propyl linked heme, the amount of observed BLH decreased when cells were heme depleted. This was seen in Figure 11a by the peak at 10.2 minutes representing the propyl linked heme. In each case the heme peak around 7.8 minutes also decreases under SA treatment. This was expected, though some residual heme is still able to be taken up due to heme impurity in the BLH samples. This result could indicate whether our BLH interact with yeast's heme uptake systems similar to heme. Because the PEG3 and PEG6 linked heme uptake is increased while propyl linked heme is decreased, PEG3 and PEG6 linked heme may act more like heme than

propyl linked heme. Though in all cases, the difference was not as drastic as seen by heme, indicating that none interacted very well with the cell's endogenous uptake systems.

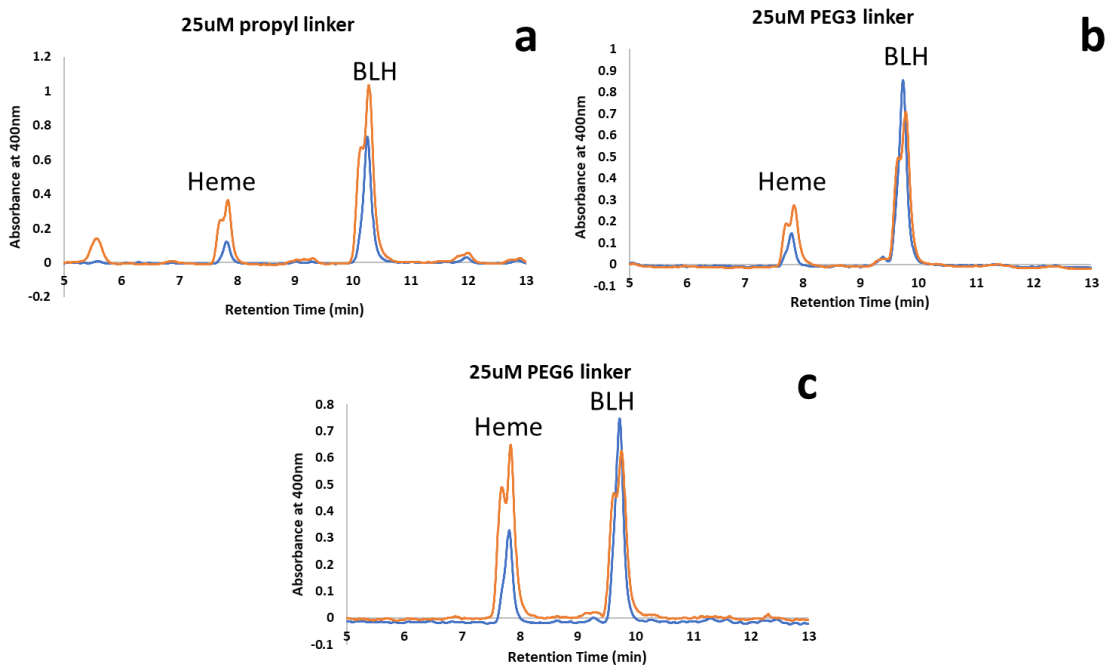

Figure 11: BLH uptake under heme deplete and replete conditions. Similar to Figure 10, yeast cells were fed BLH under heme deplete or heme replete conditions. Yeast cells were fed 25 uM BLH with either propyl (a), PEG3 (b), or PEG6 (c) linker. Yeast cells were fed BLH under heme deplete conditions, fed 500 uM SA (blue) or heme replete conditions, without SA (red).

Due to the fact that BLH uptake was not drastically improved under heme deplete conditions, we then used the over expression of a known heme uptake protein, HRG-4, to improve uptake of our BLH. This protein was discovered in *Caenorhabditis elegans* and shown to improve heme uptake when reconstituted in yeast.[15] This method helped in two ways 1) We were able to see how our BLH functioned with another known hemoprotein and 2) We were able to improve uptake of our BLH for the purposes of our enrichment method. To determine these aspects, we compared yeast cells expressing HRG-4 with

empty vector (EV) cells, fed with heme or our BLH. Heme uptake was clearly improved upon HRG-4 expression, as seen in Figure 12a, as intracellular heme was increased between EV and HRG-4 expressing cells. In Figure 12b, uptake of the propyl linked heme did not change significantly, indicating that it did not function well with HRG-4. In the traces in Figure 12b, the propyl linked heme peaks seemed unaffected by the expression of HRG-4, seen by the peak at 10.0 minutes. Finally, the uptake of PEG3 and PEG6 linked heme in Figures 12c and 12d could be seen increasing. This was based on the fact that the peaks at around 9.3 minutes and 9.7 minutes increased drastically upon HRG-4 expression. This indicated that both PEG3 and PEG6 linked heme can be taken into the cell by HRG-4. It also showed that this could be an effective method to improve uptake for the subsequent enrichment experiments.

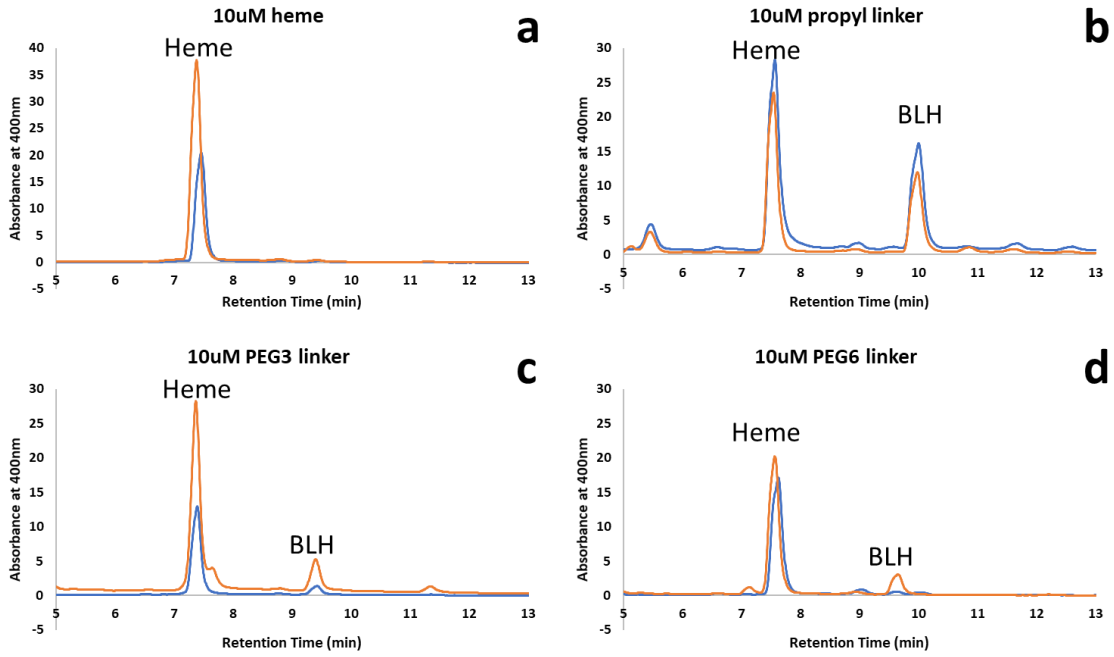

Figure 12: BLH uptake in HRG-4 expressing yeast cells. Chromatograms show heme and BLH extracted from cells lysates of yeast cells with or without HRG-4 expression. Yeast cells were fed 10 uM heme (a) or 10 uM BLH with propyl (b), PEG3 (c), or PEG6 (d) linker. Yeast cells containing an EV plasmid, lacking HRG-4, shown in blue, while those expressing HRG-4 shown in red. Both EV and HRG-4 expressing cells are grown in 0.1% galactose, causing induction of the galactose inducible promoter.

3.3 Enrichment from Cells

Once uptake of the BLH was well established, enrichment of proteins from cells was feasible. A major goal was to get as much enrichment as possible from cell while limiting background protein from non-specific binding. The more we could do this, the easier it would be to identify heme binding proteins. To achieve this, we needed to optimize various aspects of enrichment process. A large aspect of this was removing background caused from using streptavidin bound beads.

3.3.1 Optimization of Enrichment

The issue of background protein binding came about because of our use of streptavidin bound agarose covered magnetic beads. This was due to both the proteins which bound to agarose and proteins which were endogenously biotinylated. These proteins bound independent upon treatment with BLH and phosphine biotin, which prevented us from getting a clear view of protein enrichment. By 1-D SDS PAGE gel it was especially difficult to discern some background from proteins that were enriched.

To eliminate these background binding proteins, we used different methods of blocking background proteins. This included both a blocking step for endogenously biotinylated proteins and a preclearance step which removed sepharose binding proteins. To block endogenously biotinylated proteins, we added streptavidin to cell lysates. In order to determine the amount of streptavidin needed to block endogenously biotinylated proteins, we incubated lysates with varying amounts of streptavidin. Then the lysates were incubated with the streptavidin magnetic sepharose beads and eluted by boiling the beads. As seen in Figure 13, most background bands are still present after treatment with streptavidin, while two high molecular weight proteins are removed with increasing amounts of streptavidin. This is demonstrated even further when imaged by western blotting for biotin. This can be seen in Figure 14, as the two prominent endogenously biotinylated protein bands are eliminated upon addition of 50 ug or more of streptavidin.

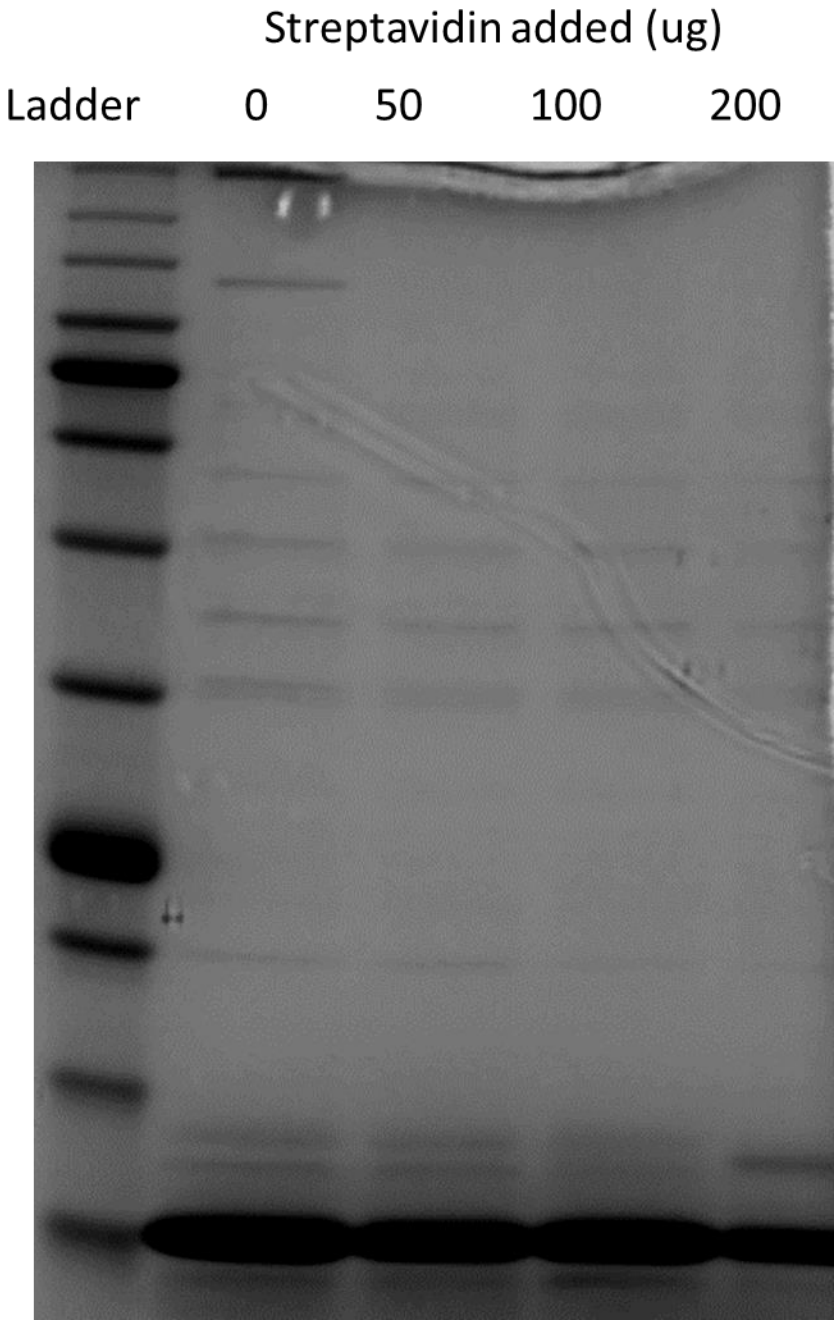

Figure 13: Streptavidin blocking shown by 1-D protein gel. 360 uL of 1 mg/mL yeast lysates were incubated with either 0, 50, 100, or 200 ug streptavidin before incubation with 50 uL of streptavidin magnetic sepharose bead slurry. Beads were then washed, and proteins eluted by SDS at 100ºC. Eluents from the beads were then run on SDS PAGE and stained with Coomassie.

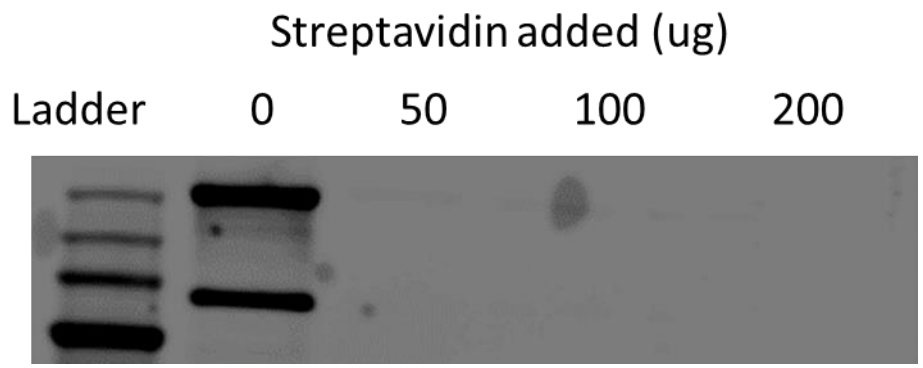

Figure 14: Streptavidin blocking shown by western blot with biotin anti-bodies. The same eluents from Figure 13 were run on SDS PAGE and then transferred to nitrocellulose membranes from western blotting. Membranes were blotted using biotin anti-bodies. Bands show two endogenously biotinylated proteins around 245kDa and 135kDa, which disappear upon streptavidin treatment.

Next, cell lysates were incubated with agarose beads to attempt to remove agarose binding proteins. Though this step may be important to the work flow of this method, no noticeable difference was observed by 1-D gel in yeast cell lysates. This step is more important to mass spectrometry based approaches.

Another issue that occurred while using the streptavidin magnetic beads was that incubation time with the lysate impacted background greatly. It was important to allow the biotinylated molecules to associate with streptavidin. However, we found that process occurred quite rapidly. When incubation time increased, more background proteins were observed in elutions. As seen in Figure 15a, the number of bands increased greatly after 30 minutes of incubation. The number of endogenously biotinylated proteins stayed constant through, observed in Figure 15b by using western blotting anti-biotin. This indicated the increasing number of bands came from non-specific binding. In order to eliminate these non-specifically binding proteins, incubation time was clearly important and was be optimized to still allow biotinylated molecules to associate.

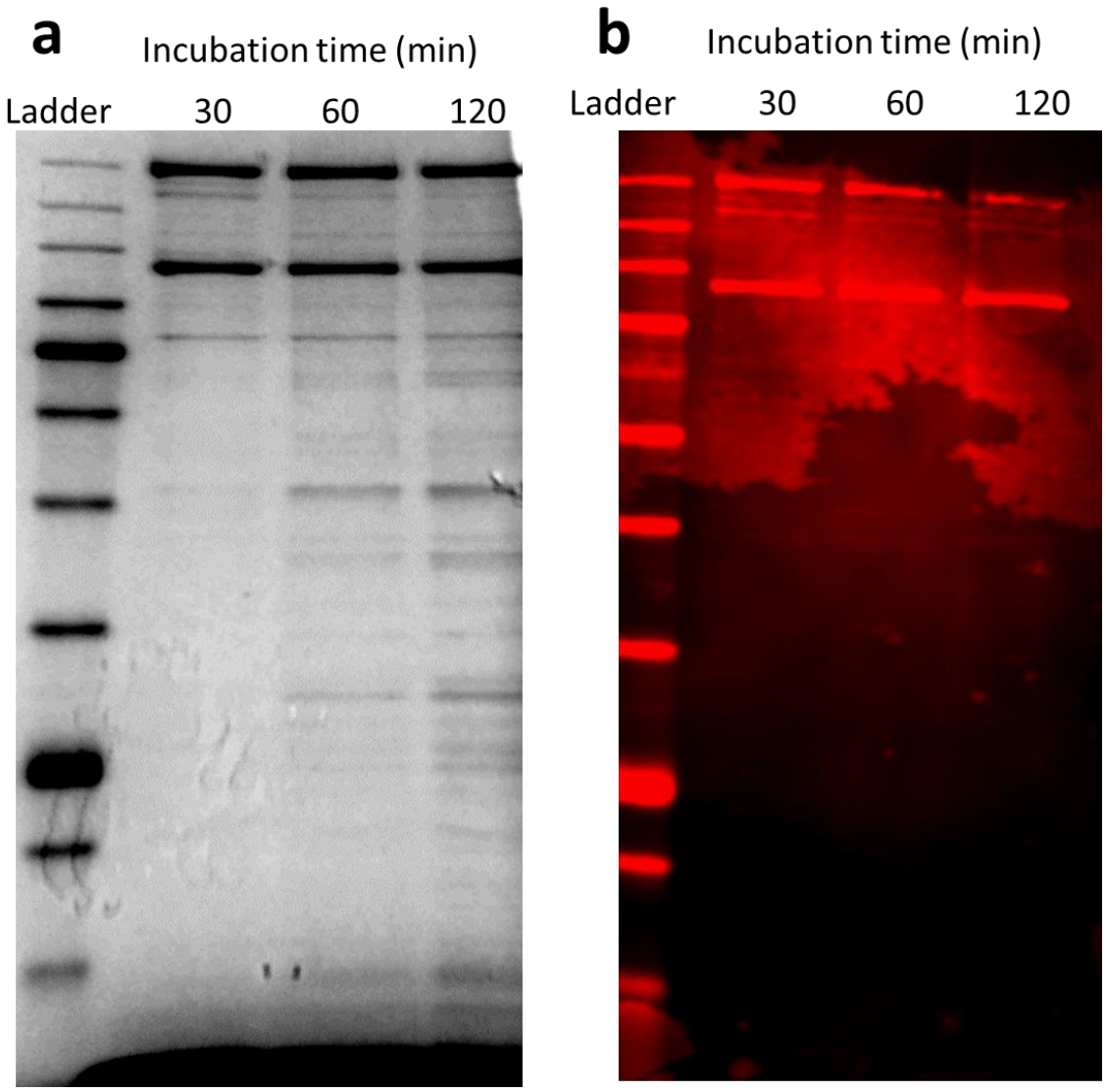

Figure 15: Determining incubation times with streptavidin magnetic beads. Wild type yeast cell lysates (360 uL, 1 mg/mL) were incubated with 50 uL of streptavidin magnetic sepharose bead slurry. This was done to determine non-specifically protein binding over time as well as time needed to associate biotinylated proteins. Beads were washed and eluted with SDS at 100ºC. Eluents are either run on SDS PAGE gel and stained with Coomassie to show all proteins bound (a) or by western blotting with anti-biotin to show biotinylated proteins associated specifically to streptavidin (b).

When boiling the streptavidin magnetic beads to elute proteins, proteins bound to the beads may elute even though they are not associated with our BLH. This is because use of SDS and boiling the samples causes all proteins bound to the protein to become

solubilized. To try to selectively elute proteins bound to our BLH, we tried to add an excess of imidazole. This method allows for the imidazole to exchange with the ligands binding to the iron of the BLH, causing the protein initially associated to dissociate. This was done to elute myoglobin enriched from solution. Either heme or our BLH analog was incubated with myoglobin and then reacted with phosphine-PEG3-biotin. This solution was then incubated with the streptavidin magnetic sepharose beads. The beads were washed and then the myoglobin bound was eluted either by boiling is SDS or with imidazole at various temperatures. As seen in Figure 16, each sample incubated with heme was unable to enrich myoglobin because it lacked the ability to bind the streptavidin magnetic beads. In each case with the BLH analog myoglobin could be seen to elute. This meant that eluting a model hemoprotein was effective by using an imidazole elution or boiling in SDS.

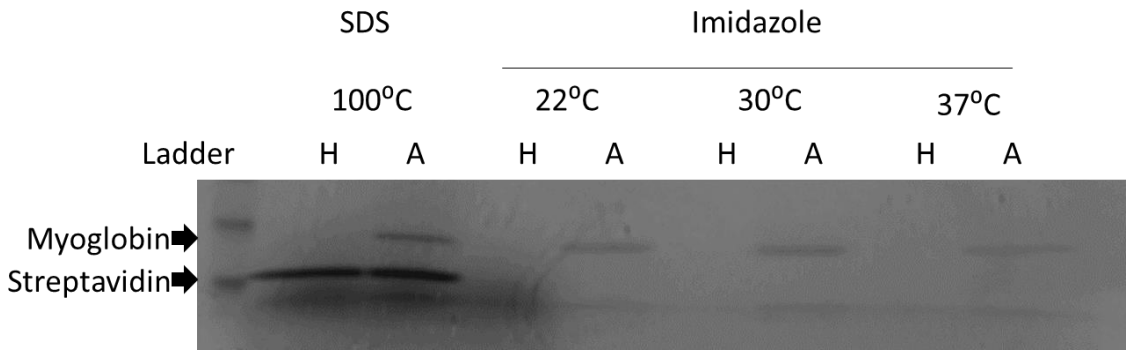

Figure 16: Comparing elution conditions from streptavidin magnetic beads with myoglobin enrichment. 0.2 mg/mL apo-myoglobin was incubated with 10 uM of either heme (H) or PEG3 linked heme analog (A) in 100 uL PBS. Then 100 uM phosphine-PEG3-biotin was reacted with both followed by incubation with 50 uL of streptavidin magnetic sepharose bead slurry. Bound myoglobin was then eluted with equal volumes of SDS at 100ºC or 1 M imidazole at 22ºC, 30ºC, or 37ºC.

3.3.2 Applications of Method

Once we had developed the method of feeding BLH and the method of hemoprotein enrichment from solution, we wanted to apply it in order to enrichment hemoproteins from cells. Enrichment was observed by 1-D SDS PAGE with relative ease. Though 1-D SDS PAGE has been effective in showing that enrichment with the BLH is possible, use of quantitative mass spectrometry would greatly improve this result. With this method, protein identification and enrichment would be determinable. From that list of proteins collected, we could then study those proteins further.

First, we wanted to know which BLH worked best at enriching proteins from cells. To do this heme deplete wild type yeast cells were fed as described above. The blocked and precleared lysates were then split and either treated with or without phosphine-PEG3-biotin. They were then incubated with streptavidin magnetic sepharose beads. Proteins bound were then eluted and run on 1-D SDS PAGE. As seen in Figure 17, background can still be seen in lanes lacking phosphine-PEG3-biotin. But in each case, the addition of phosphine-PEG3-biotin led to a darkening of the lane as well as specific bands darkening. This implies that each of BLH are able to enrich proteins from lysate, though there is still significant background visible. When comparing between BLH, it appears that the PEG3 and PEG6 linked heme is able to enrich more protein from lysates than the propyl linked heme. The fact that the propyl linked heme does not seem to enrich proteins as well is consistent with our *in vitro* characterization.

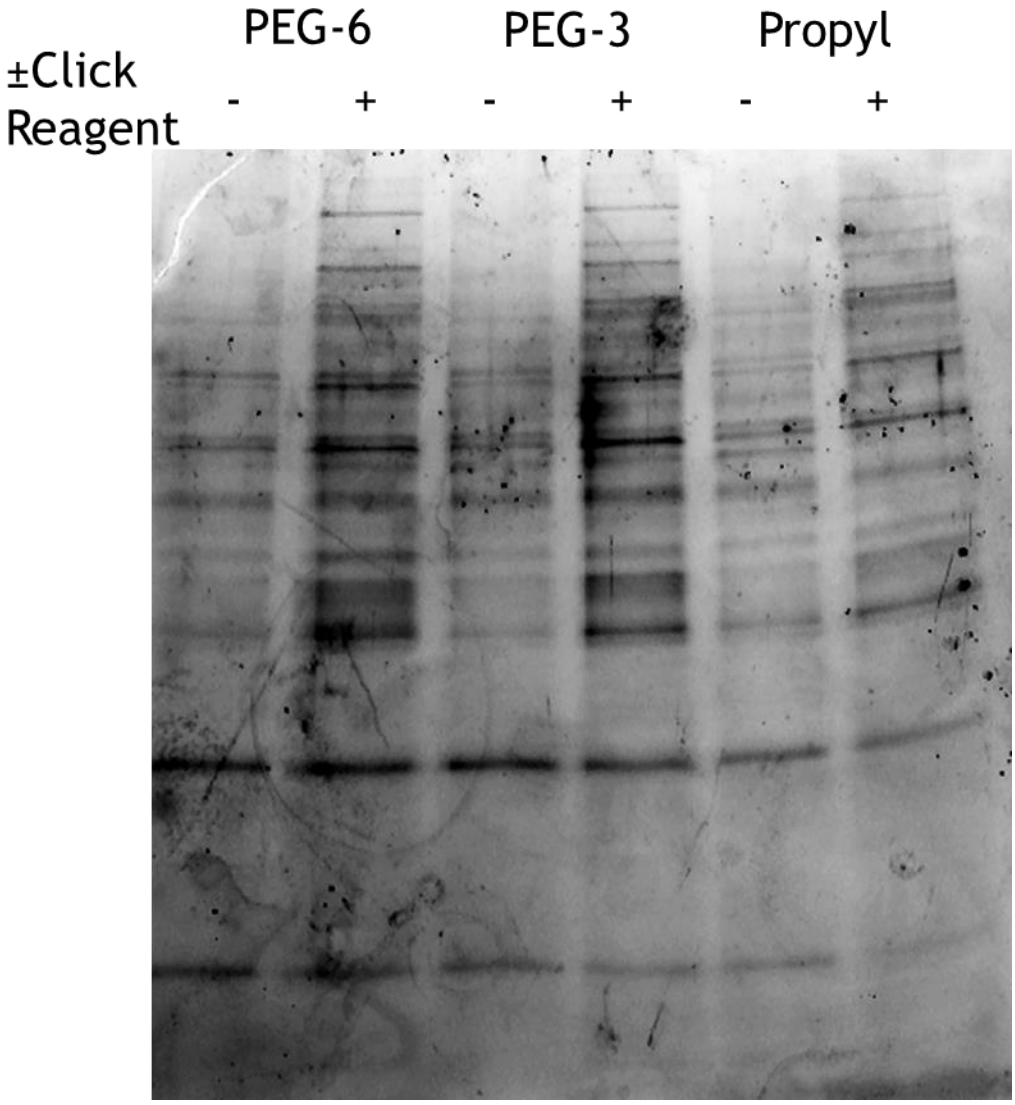

Figure 17: Proteins enriched from yeast cells fed BLH with either propyl, PEG3, or PEG6 linker. Wild type yeast cells grown up under heme deplete conditions with 500 uM succinyl acetone were fed 25 uM of each BLH. The cell lysates (360 uL, 1 mg/mL) were either incubated without (-) or with (+) 200 uM phosphine-PEG3-biotin and then enriched with 50 uL of streptavidin magnetic sepharose bead slurry. Beads were washed and eluted with an imidazole elution buffer. Eluents were then run on SDS PAGE and then stained with Sypro ruby protein stain.

Once determined that we could enrich proteins to some extent from wild type cells, we also wanted to try under the condition of HRG-4 over expression to improve BLH uptake. In this experiment, we fed HRG-4 expressing cells either heme or BLH. This way

BLH could be reacted with phosphine-PEG3-biotin while heme could not be. The similar blocking and preclearance steps were taken to remove background. In this case both lysates were reacted with phosphine-PEG3-biotin and then incubated with streptavidin magnetic sepharose beads. The eluted proteins can be seen in Figure 18. Under the lane for cells fed heme, the background binding proteins can be seen, as heme cannot react with phosphine-PEG3-biotin and can therefore not enrich proteins bound. As for those fed with PEG3 linked heme, similar background can be seen plus the addition of a few bands. This result clearly shows that proteins can be enriched from lysates by this method.

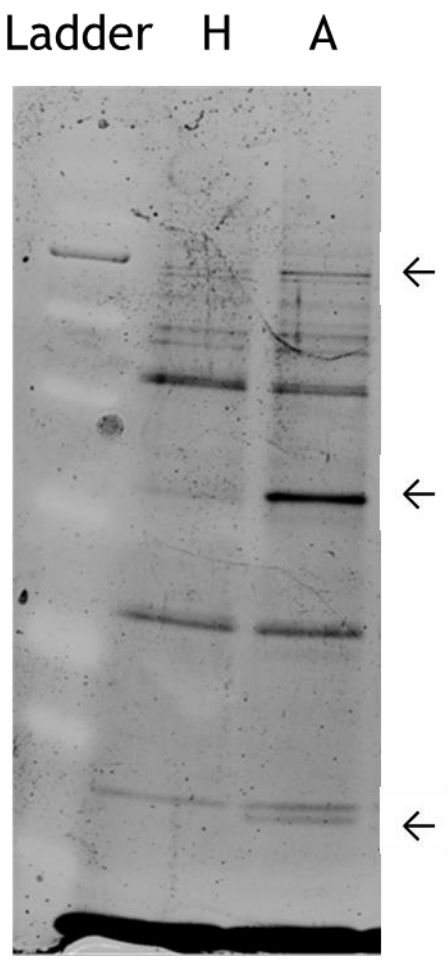

Figure 18: Proteins were enriched from cells expressing HRG-4 using BLH. Yeast cells were fed 25 uM of either heme (H) or PEG3 linked heme analog (A). Both lysates were reacted with 200 uM phosphine-PEG3-biotin and then enriched with 50 uL streptavidin magnetic bead slurry. Beads were washed and eluted into SDS at 100ºC. Eluents were run on SDS PAGE and stained with Sypro ruby protein stain. Arrows indicate proteins visibly enriched in PEG3 linked heme samples compared to heme samples.

CHAPTER 4. CONCLUSION AND FUTURE WORK

4.1 Conclusion and Future Work

Heme is an important cofactor found everywhere within the cell, essential to virtually every aerobic organism. Despite being ubiquitous, little is known about heme trafficking and homeostatic factors. In order to build a better picture of these aspects of heme, we have designed a tool that can help in the determination of heme binding proteins. Through the use of bioorthogonal azide labeling, we were able to modify heme in a way that did not significantly impact the electronics of heme or its ability to bind model hemoproteins. We were also able to show that some of the bioorthogonally labelled heme (BLH) were able to act in a heme-like way with known hemoproteins, such as myoglobin and HRG-4. After optimizing uptake of the BLH and the method of enrichment, we were able to see enrichment of some proteins from cells fed BLH.

In many cases this enrichment was not significantly greater the protein background from using streptavidin magnetic beads, observed by 1-D gel. This is a portion of the work that still requires improvement in order to implement it. We believe that little enrichment compared to the background observed could be due to many factors including limitations to the click reaction, proteins dissociating from BLH under reaction conditions, and simply low hemoprotein abundance. To successfully implement this method, further optimization of the click reaction conditions would be necessary along with limiting background binding proteins further. Once this is done mass spectrometric analysis could be done to identify proteins enriched through this method. The proteins identified could then further be studied to determine their impact upon heme homeostasis.

Once this method is optimized to effectively enrich known and novel hemoproteins from cells, it could be extended to study the heme binding proteome in a variety of different genetically intractable organisms. Many pathogens contain heme uptake systems which could be further studied using these methods of hemoprotein enrichment. A few examples of model organisms that this method could be applied in would be *Candida albicans* and *Mycobacterium tuberculosis*, in which heme uptake has already been characterized to some extent.[33,34] The use of our BLH could improve our understanding of heme homeostasis in these contexts as well.

APPENDIX A. METHODS

Table 1: Description of plasmids used

Plasmids Used	Description
pRS316-GAL-EV	Empty vector plasmid with gal inducible promoter under ura selection
pYes-Dest52-HRG4	HRG4 expressing plasmid with gal inducible promoter under ura selection

Reagents and Materials Used. N,N'-dicyclohexylcarbodiimide (DCC, 99%) was acquired from Alfa Aesar Cas # 538-75-0, 1-hydroxybenzotriazole hydrate wetted with not less than 20% water (HOBt, 97%) was acquired from Chem Impex International Inc Cas # 123333-53-9, 3-azidopropylamine (>95%) was acquired from Tokyo Chemical Industry Cas # 88192-19-2, 11-azido-3,6,9-trioxaundecan-1-amine (azido-PEG3-amine, >95%) was acquired from Tokyo Chemical Industry Cas # 134179-38-7, O-(2-Aminoethyl)-O′-(2-azidoethyl) pentaethylene glycol (azido-PEG6-amine, >90% oligomer purity) was acquired from Sigma Aldrich Cas # 957486-82-7, myoglobin from equine skeletal muscle (95-100%) was obtained from Sigma Aldrich Cas # 100684-32-0, streptavidin was acquired from VWR Cas # 97062-810, EZ-link™ phosphine-PEG3-biotin was acquired from Thermo Scientific Cat # 88901, sepharose® 4B (sepharose bead slurry) was acquired from Sigma Aldrich Cas # 9012-36-6, PD SpinTrap™ G-25 was acquired from GE Healthcare Cat #28918004, Streptavidin Mag Sepharose™ was acquired from GE Healthcare Cat # 28985799

Synthesis of Heme Analogs. A solution of hemin-cl (13 mg, 2 mmoles, 1 eq) in dimethyl formamide with 5% pyridine (2 mL, 10 mM) was prepared followed by the addition of

DCC (4 mg, 2 mmoles, 1 eq) and HOBt (3 mg, 2 mmoles, 1 eq) in a 15 mL centrifuge tube. Either 3-azido-1-propanmine (2 uL, 2 mmoles, 1 eq), azido-PEG3-amine (4.36 uL, 2 mmoles, 1 eq), or azido-PEG6-amine (7 uL, 2 mmoles, 1 eq) was added and left to agitate on nutating mixer for 48-72 hours covered in aluminum foil at room temperature. Reaction progress was checked by analytical scale HPLC described below.

Preparatory HPLC Purification. The reaction mix was diluted 1:5 in 50% acetonitrile/50% water with 0.1% trifluoroacetic acid. 5 mL was then run on C3 preparatory scale HPLC. The mobile phase was run on a gradient from 50-52.5% acetonitrile with 0.1% trifluoroacetic acid over 30 minutes at 5 mL/minute, followed by sustained 52.5% acetonitrile with 0.1% trifluoroacetic acid at 5mL/minute for 15 minutes. The column was then washed for 20-30 minutes with 100% acetonitrile. Chromatograms were monitored at 400 nm, 280 nm, and 220 nm. Fractions were collected every two minutes. Fractions were analyzed by analytical scale HPLC described below. Significant fractions were then lyophilized and resuspended in DMSO. Heme eluted 19-21 minutes, PEG3 linked heme eluted 22-25 minutes, and 2x(PEG3-azide) linked heme eluted 26-29 minutes.

Analytical HPLC Analysis. Samples were diluted into 50% acetonitrile/50% water with 0.1% trifluoroacetic acid. 20 uL of the diluted sample was then run on C18 analytical scale HPLC. The mobile phase was run on a gradient from 40-55% acetonitrile with 0.1% trifluoroacetic acid, over 15 minutes, at 1 mL/minute. The column was then washed for 10 minutes with 100% acetonitrile. Chromatograms were monitored at 400 nm, 280 nm, and 220 nm.

Yeast Strains, Genetic Manipulations, and Media. *S. cerevisiae* strains used were derived from BY4741. Yeast transformations were done following a lithium acetate procedure.[35] Strains were grown at 30°C on synthetic complete media (SC) supplemented with glucose. Heme deplete cells were grown on SC media with 500 uM SA. Strains containing plasmids with galactose inducible promoters were grown with SC media drop out media without uracil to maintain selection, supplemented with 2% raffinose and 0.1% galactose.

Apo-myoglobin Preparation. Apo-myoglobin stocks were made extracting heme from myoglobin. 5 mL of 15-20 mg/mL myoglobin in deionized water was added dropwise to 250 mL acid acetone (5 mM HCl) at 4°C in a 250 mL flask. The suspension was then centrifuged at 3200 g at 4°C for 10 minutes and supernatant removed. If the pellet was still red, then it was resuspended in 50 mL fresh acid acetone at 4°C followed by centrifugation at 3200 g and removal of supernatant. The final precipitate was then resuspended in 4 mL cold deionized water and dialyzed at 4°C in 500 mL 1xPBS at pH 7.4. Precipitate was then pelleted, and supernatant was used for subsequent experiments.[36]

Myoglobin Enrichment Conditions. In 100 uL of 1x PBS, apo-myoglobin stocks (19.6 mg/mL in PBS) were diluted to 0.2 mg/mL. Heme or BLH (10 mM in DMSO) was added to a final concentration of 10 uM. Then phosphine-PEG3-biotin (10 mM in DMSO) was added to a final concentration of 100 uM. Samples were then incubated at 37°C for 3 hours. 50 uL of streptavidin magnetic sepharose bead slurry were equilibrated with 200-300 uL of lysis buffer. The myoglobin/BLH solution was then added to 50 uL streptavidin magnetic sepharose bead slurry and left at room temperature agitating on nutating mixer

for 30 minutes. Solution was removed, and beads were washed 3 times with 750 uL of 1xPBS for 5 minutes per wash. Samples were eluted into 50 uL of 1x SDS (5x SDS that was diluted into PBS) by heating to 100°C for 10 minutes. In some cases, an imidazole elution was done by adding 50 uL of 1 M imidazole in lysis buffer (10mM NaPi, 50mM NaCl, 5mM EDTA, 0.1% Triton x-100, pH 7.4 after imidazole addition) and incubating for 15 minutes at room temperature. 5x SDS was then added to imidazole eluted samples and denatured at 100°C for 5 minutes. Eluents were then run on SDS PAGE and stained with Coomassie.

Determination of Heme and BLH Uptake. Cells were grown overnight at 30°C to around 1 OD/mL. The cells were then pelleted and reinoculated at 10 OD/mL in 1.5 mL fresh media with 10- 25 uM of heme or BLH for 3 hours. Cells were then pelleted in a 1.5 mL centrifuge tube. Cell pellets were washed with washing buffer (0.1% tween 20, 2% bovine serum albumin in 2x PBS). They were then lysed into 50 uL lysis buffer (10mM NaPi, 50mM NaCl, 5mM EDTA, 0.1% Triton x-100, pH 7.4) with about 1 pellet volume of ZrO beads using a bullet blender, at speed 8 for 3 minutes. Cell debris was removed by centrifugation and lysates collected. Heme and BLH were then extracted from lysates by adding cell lysates dropwise to cold acid acetone (5 mM HCL). Protein precipitates were then pelleted by centrifuging at 14.0 G for 3 minutes. Extracts were then run on analytical scale HPLC described above.

Enrichment from Yeast Cell Lysates. Cells were grown overnight at 30°C to around 1 OD/mL. The cells were then pelleted and reinoculated at 10 OD/mL in 1.5 mL fresh media with 10-25 uM heme or BLH for 3 hours. Cells were then pelleted in a 1.5 mL centrifuge

tube. The cell pellet was then lysed into 100 uL lysis buffer (10mM NaPi, 50mM NaCl, 5mM EDTA, 0.1% Triton x-100, pH 7.4) with 1x ProteaseArrest and 1 mM phenylmethylsulfonyl fluoride with about 1 pellet volume of ZrO beads with a bullet blender at speed 8 for 3 minutes at 4°C. Cell debris was then removed by centrifuging at 14 G at 4°C and protein concentrations were calculated by Bradford assay. Lysates were then diluted to 1 mg/mL protein concentration in 700 uL. 10 uL of streptavidin (20 mg/mL stock in PBS stored at 4°C) was then added and left at room temperature for 30 minutes. Then 10 uL of 1.8 mM biotin in ethanol (stored at 4°C) was added and left at room temperature for 30 minutes. The samples were then split into 360 uL aliquots. One aliquot was treated with 7.2 uL of phosphine-PEG3-biotin (10 mM in DMSO), while the other was treated with 7.2 uL DMSO. 240 uL of Sepharose® 4B bead slurry were then washed 3x with 400 uL lysis buffer for 10 seconds at 0.1 G. Both lysates were then incubated with the 240 uL of sepharose® 4B bead slurry at 37°C for 3 hours. sepharose® 4B beads were then removed and lysates were run through PD SpinTrap™ G-25 Columns following recommended protocol to remove excess phosphine-PEG3-biotin and biotin. 50 uL of streptavidin magnetic sepharose bead slurry were equilibrated with 200-300 uL of lysis buffer. Samples were then incubated with the 50 uL of streptavidin magnetic sepharose bead slurry for 30 minutes. Flow through was saved as loading control. Beads were collected and washed 3 times with 750 uL lysis buffer for 5 minutes per wash while agitating on nutating mixer. 50 uL of 1x SDS (5x SDS diluted into lysis buffer) was then added to beads and heated to 100°C for 5 minutes to elute protein. In some cases, an imidazole elution was done by adding 50 uL of 1 M imidazole in lysis buffer (10mM NaPi, 50mM NaCl, 5mM EDTA, 0.1% Triton x-100, pH 7.4 after imidazole addition) and

incubating for 15 minutes at room temperature. 5x SDS was then added to imidazole eluted samples and denatured at 100°C for 5 minutes. Eluents were then run on SDS PAGE and stained with Sypro ruby protein stain.

REFERENCES

1. Severance, S. & Hamza, I. Trafficking of heme and porphyrins in metazoa. *Chem. Rev.* **109,** 4596–4616 (2009).
2. Reddi, A. R. & Hamza, I. Heme Mobilization in Animals: A Metallolipid's Journey. *Acc. Chem. Res.* **49,** 1104–1110 (2016).
3. Yuan, X. *et al.* Regulation of intracellular heme trafficking revealed by subcellular reporters. *Proc. Natl. Acad. Sci. U. S. A.* **113,** E5144-52 (2016).
4. Hanna, D. A. *et al.* Heme dynamics and trafficking factors revealed by genetically encoded fluorescent heme sensors. *Proc. Natl. Acad. Sci.* **113,** 7539–7544 (2016).
5. Hanna, D. A., Martinez-Guzman, O. & Reddi, A. R. Heme Gazing: Illuminating Eukaryotic Heme Trafficking, Dynamics, and Signaling with Fluorescent Heme Sensors. *Am. Chem. Soc. Biochem.* **56,** 1815–1823 (2017).
6. Connor, J. R., Snyder, B. S., Beard, J. L., Fine, R. E. & Mufson, E. J. Regional Distribution of Iron and Iron-Regulatory Proteins in the Brain in Aging and Alzheimer ' s Disease. **31,** 327–335 (1992).
7. Tolosano, E. Heme in pathophysiology : a matter of scavenging , metabolism and trafficking across cell membranes. **5,** 1–24 (2014).
8. Kumar, S. & Bandyopadhyay, U. Free heme toxicity and its detoxification systems in human. *Toxicol. Lett.* **157,** 175–188 (2005).
9. Shen, J. *et al.* Iron metabolism regulates p53 signaling through direct Heme-p53 interaction and modulation of p53 localization, stability, and function. *Cell Rep.* (2014). doi:10.1016/j.celrep.2014.02.042
10. Schipper, H. M., Song, W., Zukor, H., Hascalovici, J. R. & Zeligman, D. Heme oxygenase-1 and neurodegeneration: Expanding frontiers of engagement. *Journal of Neurochemistry* (2009). doi:10.1111/j.1471-4159.2009.06160.x
11. Atamna, H., Killilea, D. W., Killilea, A. N. & Ames, B. N. Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 14807–12 (2002).
12. Todd, D. J. Erythropoietic protoporphyria. *Br. J. Dermatol.* **131,** 751–766 (1994).
13. Brenner, D. a *et al.* A molecular defect in human protoporphyria. *Am. J. Hum. Genet.* **50,** 1203–10 (1992).
14. Chiabrando, D., Mercurio, S. & Tolosano, E. Heme and erythropoieis: More than a structural role. *Haematologica* **99,** 973–983 (2014).
15. Yuan, X., Protchenko, O., Philpott, C. C. & Hamza, I. Topologically conserved residues direct heme transport in HRG-1-related proteins. *J. Biol. Chem.* **287,** 4914–4924 (2012).
16. Hanker, J. S. & Rabin, A. N. Color reaction streak test for catalase-positive microorganisms. *J. Clin. Microbiol.* **2,** 463–464 (1975).
17. Kathiresan, M., Martins, D. & English, A. M. Respiration triggers heme transfer

from cytochrome c peroxidase to catalase in yeast mitochondria. *Proc. Natl. Acad. Sci.* **111,** 17468–17473 (2014).

18. Bracken, C. S., Baer, M. T., Abdur-Rashid, A., Helms, W. & Stojiljkovic, I. Use of heme-protein complexes by the Yersinia enterocolitica HemR receptor: Histidine residues are essential for receptor function. *J. Bacteriol.* **181,** 6063–6072 (1999).

19. Asuthkar, S., Velineni, S., Stadlmann, J., Altmann, F. & Sritharan, M. Expression and characterization of an iron-regulated hemin-binding protein, HbpA, from Leptospira interrogans serovar Lai. *Infect. Immun.* **75,** 4582–4591 (2007).

20. Wang, N. *et al.* Proteomic analysis of putative heme-binding proteins in Streptococcus pyogenes. *Metallomics* **6,** 1451–1459 (2014).

21. Chen, W., Smeekens, J. M. & Wu, R. Systematic and site-specific analysis of N-sialoglycosylated proteins on the cell surface by integrating click chemistry and MS-based proteomics. *Chem. Sci.* **6,** 4681–4689 (2015).

22. Seo, Y. H. & Carroll, K. S. Facile synthesis and biological evaluation of a cell-permeable probe to detect redox-regulated proteins. *Bioorganic Med. Chem. Lett.* **19,** 356–359 (2009).

23. Nessen, M. A. *et al.* Selective enrichment of azide-containing peptides from complex mixtures. *J. Proteome Res.* (2009). doi:10.1021/pr900257z

24. Saxon, E. Cell Surface Engineering by a Modified Staudinger Reaction. *Science (80-.).* (2000). doi:10.1126/science.287.5460.2007

25. Dunetz, J. R., Magano, J. & Weisenburger, G. A. Large-Scale Applications of Amide Coupling Reagents for the Synthesis of Pharmaceuticals. *Organic Process Research and Development* (2016). doi:10.1021/op500305s

26. Köhn, M. & Breinbauer, R. The Staudinger ligation - A gift to chemical biology. *Angewandte Chemie - International Edition* (2004). doi:10.1002/anie.200401744

27. Agard, N. J., Baskin, J. M., Prescher, J. A., Lo, A. & Bertozzi, C. R. A comparative study of bioorthogonal reactions with azides. *ACS chemical biology* (2006). doi:10.1021/cb6003228

28. Reddie, K. G., Seo, Y. H., Muse Iii, W. B., Leonard, S. E. & Carroll, K. S. Molecular BioSystems A chemical approach for detecting sulfenic acid-modified proteins in living cellswz. (2008). doi:10.1039/b719986d

29. Klomsiri, C. *et al.* Chapter 3 - Use of Dimedone-Based Chemical Probes for Sulfenic Acid Detection: Evaluation of Conditions Affecting Probe Incorporation into Redox-Sensitive Proteins. doi:10.1016/S0076-6879(10)73003-2

30. Weber, P.C., Ohlendorf, D.H, Wendolski, J.J., Salemme, F. R. Structural Origins of High-Affinity Biotin Binding to Streptavidin Author (s): Patricia C . Weber , D . H . Ohlendorf , J . J . Wendoloski and F . R . Salemme Published by : American Association for the Advancement of Science Stable URL : http://www.jst. *Am. Assoc. Adv. Sci.* **243,** 85–88 (2016).

31. Protchenko, O., Rodriguez-Suarez, R., Androphy, R., Bussey, H. & Philpott, C. C.

A screen for genes of heme uptake identifies the FLC family required for import of FAD into the endoplasmic reticulum. *J. Biol. Chem.* **281,** 21445–21457 (2006).

32. Protchenko, O. *et al.* Role of PUG1 in inducible porphyrin and heme transport in Saccharomyces cerevisiae. *Eukaryot. Cell* **7,** 859–871 (2008).

33. Contreras, H., Chim, N., Credali, A. & Goulding, C. W. Heme uptake in bacterial pathogens. *Curr. Opin. Chem. Biol.* **19,** 34–41 (2014).

34. Tullius, M. V. *et al.* Discovery and characterization of a unique mycobacterial heme acquisition system. *Proc. Natl. Acad. Sci.* **108,** 5051–5056 (2011).

35. Gietz, R. D. & Schiestl, R. H. Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* **7,** 253–263 (1991).

36. Franca, A., Fanelli, M. R. R. & Antonini, E. Preparation and Properties of Apohemoglobin and Reconstituted Hemoglobins. **76,** 72–87 (1981).